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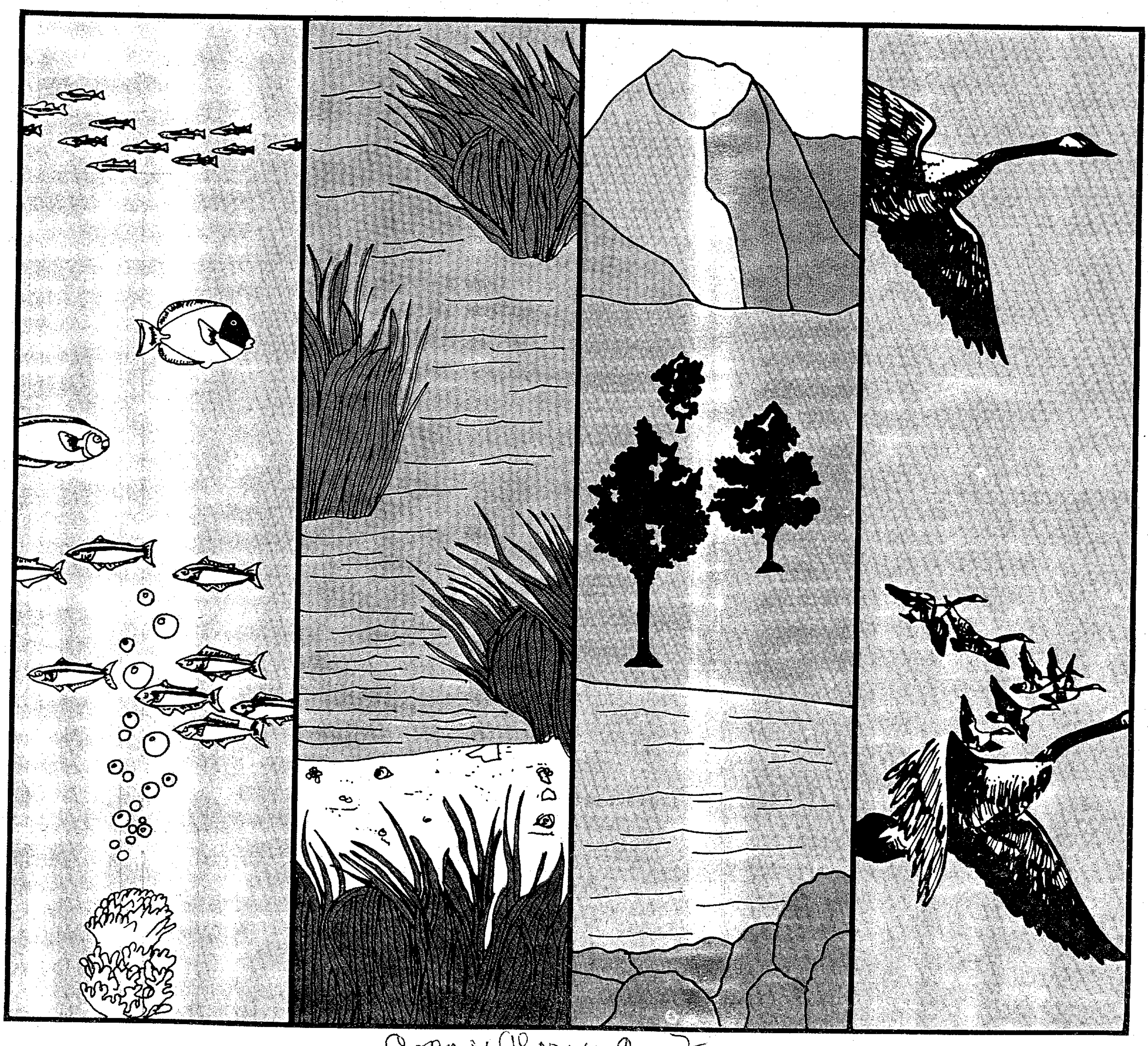
Acute Toxicity Test for Freshwater Invertebrates



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HAZARD EVALUATION DIVISION

STANDARD EVALUATION PROCEDURE

ACUTE TOXICITY TEST FOR FRESHWATER INVERTEBRATES

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STANDARD EVALUATION PROCEDURE

PREAMBLE

This Standard Evaluation Procedure (SEP) is one of a set of guidance documents which explain the procedures used to evaluate environmental and human health effects data submitted to the Office of Pesticide Programs. The SEPs are designed to ensure comprehensive and consistent treatment of major scientific topics in these reviews and to provide interpretive policy guidance where appropriate. The Standard Evaluation Procedures will be used in conjunction with the appropriate Pesticide Assessment Guidelines and other Agency Guidelines. While the documents were developed to explain specifically the principles of scientific evaluation within the Office of Pesticide Programs, they may also be used by other offices in the Agency in the evaluation of studies and scientific data. The Standard Evaluation Procedures will also serve as valuable internal reference documents and will inform the public and regulated community of important considerations in the evaluation of test data for determining chemical hazards. I believe the SEPs will improve both the quality of science within EPA and, in conjunction with the Pesticide Assessment Guidelines, will lead to more effective use of both public and private resources.

John W. Melone, Director
Hazard Evaluation Division

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ACUTE TOXICITY TEST FOR FRESHWATER INVERTEBRATES

I. INTRODUCTION

A. When Required

Acute toxicity testing on freshwater invertebrates is required to support registration of manufacturing-use pesticide products and end-use pesticide products intended for outdoor application.

B. Purpose

Acute toxicity studies on freshwater invertebrates determine the lethal concentration (LC_{50}) or effect concentration (EC_{50}) of a chemical which will kill or immobilize, respectively, fifty percent of the test population in 48 to 96 hours. These acute tests have attained broad acceptance among environmental toxicologists as relatively rapid, uncomplicated, inexpensive, and statistically reliable methods for assessing immediate, short-term, adverse effects of chemicals on freshwater invertebrates.

The Ecological Effects Branch regularly requires that results of one freshwater invertebrate acute toxicity test be submitted to support the registration of a pesticide. The data from this test are used:

- To establish acute toxicity levels of the active ingredient to nontarget freshwater invertebrates;
- To assess potential impact to invertebrates by comparing toxicity information with measured or estimated pesticide residues in the freshwater environment;
- To provide support for precautionary label statements that will minimize adverse effects to freshwater invertebrates when the the pesticide is used according to directions; and
- To indicate the need for further laboratory testing and/or field studies.

C. Test Material

1. Technical Grade

Tests must be conducted with the technical grade of the active ingredient. If more than one active ingredient constitutes a technical product, then the technical grade of each active ingredient must be tested separately.

2. End-Use Product

In addition to technical product testing, the applicant may be required to test the end-use product as well if:

- The end-use product will be introduced directly into an aquatic environment when used as directed;
- The freshwater invertebrate LC_{50} (or EC_{50}) of the technical grade of the active ingredient is equal to or less than the expected environmental concentration in the freshwater environment when the end-use product is used as directed;
- An ingredient of the formulated end-use product is expected to enhance the toxicity of the end-use product beyond that expected from the active ingredient(s) alone; or
- The technical product is insoluble in water but the formulated product is soluble in water. In this situation, the test design should include a control where organisms are exposed to just the carriers and/or inert ingredients.

II. MATERIALS AND METHODS: TESTING STANDARDS/DATA ACCEPTABILITY

A. Recommended Protocols

Because the acute test is an established technique for assessing toxicity of a chemical to aquatic invertebrate species, much of the methodology for performing these studies, as well as the procedures for statistical analysis of results, have been carefully outlined and documented in the published literature. Notably, the information to be discussed in this Standard Evaluation Procedure (SEP) is presented in greater detail in the following references:

Committee on Methods for Toxicity Tests with Aquatic Organisms. 1975. Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians. U.S. Environmental Protection Agency, Ecol. Res. Series, EPA 660/375-009. 61 pp.

American Society for Testing Materials. 1980. Standard Practice for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians. E 729-80. Published by ASTM Committee on Standards, 1916 Race Street, Philadelphia, PA, 19103.

Peltier, William. 1978. Methods for Measuring the Acute Toxicity of Effluents to Aquatic Organisms. U.S. Environmental Protection Agency, Ecol. Res. Series, EPA 600/4-78-012. 52 pp.

U.S. Environmental Protection Agency. Pesticide Assessment Guidelines Subdivision E. Hazard Evaluation: Wildlife and Aquatic Organisms.

These referenced protocols are presented as flexible guidance to help researchers design scientific protocol and to help the reviewer validate studies. It is important to recognize that freshwater invertebrate tests are validated as to whether they provide scientifically sound information on the acute toxicity of the test material to freshwater invertebrates and whether the results of the study will fulfill guideline requirements. This is more important than whether a study completely conforms to referenced protocols. It is sometimes necessary to alter the procedures presented in published protocols to meet the characteristics of the chemical or test organisms used.

The static test is the standard technique for obtaining LC_{50} or EC_{50} values for aquatic invertebrates; however, flow-through testing may be needed when toxicants are highly volatile or otherwise unstable in the aqueous environment, or when a chemical has a high biochemical oxygen demand. The information as it is presented below will focus on static testing protocols. Specific references to acceptable flow-through methods are indicated when necessary.

B. Test Organisms

1. Acceptable Species

The preferred test species for the aquatic invertebrate acute study is Daphnia magna. Daphnia were chosen on the basis of their past use in toxicity testing and known susceptibility to chemical exposure. Other acceptable species include:

Daphnia pulex	
Amphipods	(Gammarus lacustris, G. fasciatus, or G. pseudolimnaeus)
Mayflies	(Baetis spp. or Ephemerella spp.)
Mayflies	(Hexagenia limbata or H. bilineata)
Stoneflies	(Pteronarcys spp.)
Midges	(Chironomus spp.)

2. Size/Age/Physical Condition

All organisms in a test should be approximately the same size and age. Immature organisms should be used. Daphnids should be in the first instar (less than 24 hours old). Amphipods, stoneflies, and mayflies should be in the second instar; and midges should be in the second or third instar.

3. Source/Acclimation

All organisms must be from the same source. This may include laboratory or commercial stocks. Animals captured in the wild are acceptable provided they meet the requirements pertaining to physical condition and age/size criteria mentioned above. Organisms captured via chemical treatment must not be used. When test animals are brought into the laboratory, they should be quarantined for at least seven days and acclimated to study conditions for at least one week prior to testing.

Test organisms must be observed prior to testing for signs of disease, stress, physical damage, and mortality. Injured, dead, and abnormal individuals must be discarded. Organisms must not be used if they appear to be diseased or stressed or if more than 3% die during the 48 hours immediately prior to testing.

Daphnids from cultures in which ephippia are being produced should not be used. Young daphnids should be from the fourth or later brood of a given parent.

If possible, feeding of the organisms should be limited to the time just prior to testing.

C. Test System

1. Source of Dilution Water

Whenever possible, soft, reconstituted water should be used for freshwater studies. Reconstituted water should be aged one or two weeks and intensely aerated prior to use. Detailed descriptions of acceptable procedures for preparing diluent are found in the protocols by the American Society for Testing Materials (1980) or the Committee on Methods for Toxicity Tests with Aquatic Organisms (1975).

A natural dilution water with a hardness of 40 to 48 mg/L as $CaCO_3$ can be used in lieu of reconstituted water. If possible, natural dilution water should be obtained from an uncontaminated well, spring, or surface water source. Dechlorinated water should not be used because removal of chlorine is rarely complete and residual chlorine can be quite toxic to aquatic organisms.

The dilution water must be able to support the test animals without stress. Organisms should be able to survive, grow, and reproduce satisfactorily in acceptable diluent.

2. Temperature

The recommended test temperature for Daphnia is 20°C. Amphipods and mayflies (Baetis spp. and Ephemerella spp.) should be

tested at 17°C and midges and mayflies (Hexagenia spp.) at 22°C. Testing of stoneflies should be performed at 12°C.

Testing facilities should have a constant temperature area or a recirculating water bath for the test vessels.

3. Test Vessels

Test containers should be constructed from welded stainless steel or glass. Small organisms can be exposed in 3.9 liter (1 gallon) wide mouth glass jars containing 2 to 3 liters of solution. Most static tests with daphnids and midge larvae are performed in 250 ml glass beakers containing 200 mls of test solution. Beakers should be covered to prevent evaporation.

If test vessels are constructed from materials other than glass or stainless steel, solutions must be analyzed to determine exact toxicant concentrations. Past studies have shown that some test vessel materials (e.g., polyethylene) can adsorb residues of the pesticide being tested.

The metering system chosen for flow-through studies must reproducibly supply appropriate toxicant concentrations at a consistent flow rate. Metering systems should be calibrated before and after each study and checked twice daily during the test period. Flow rates should be five to ten volume additions per 24-hours. Systems should be constructed so that the organisms are not stressed by turbulence.

4. Photoperiod

A 16-hour light and an 8-hour dark photoperiod with a 15- to 30-minute transition period between light and dark is recommended.

5. Loading

The size of the test container should be such that the loading factor (test organism mass per volume of test solution) is no greater than 0.8 g/L in static tests performed at or below 17°C. At higher temperatures, a loading of 0.5 g/L is acceptable. For flow-through tests, the loading should be no greater than 1 g/L of solution passing through the chamber in 24-hours, and must not exceed 10 g/L at any time at or below 17°C or 5 g/L at higher temperatures.

6. Solvents

Whenever possible, the toxicant should be introduced into the test solution without the use of solvents other than water. If alternative solvents are necessary, they should be used sparingly,

not to exceed 0.5 ml/L in any static test solution and 0.1 ml/L under flow-through conditions. The following solvents are preferred:

dimethyl formamide
triethylene glycol
methanol
acetone
ethanol

D. Test Design

1. Test Levels

Initially, range finding tests may be necessary to define concentrations of the toxicant needed for definitive studies. If results from a range finding study indicate a low toxicity for the chemical, a definitive test need not be performed. However, it must be determined that the chemical will have an LC_{50}/EC_{50} greater than 100 mg/L, by exposing at least 30 individuals to a concentration of 100 mg/L or greater.

Definitive acute toxicity tests normally are designed to include one or more control groups and a geometric series of at least five toxicant concentrations to be tested. Each designated treatment group should be exposed to a concentration of toxicant that is at least 60% of the next highest concentration.

2. Number of Test Animals

In definitive tests, at least 20 test organisms should be exposed to each treatment level. Treatment groups can be divided into two or more containers. All organisms must be randomly assigned to test vessels.

3. Controls

Each test requires a concurrent control using the same dilution water and same number of organisms per test level. If any solvent other than water is used, a solvent control should be established. The highest concentration of the solvent that was added to any of the test chambers should be used in the control.

A test is not acceptable if more than 10% of the control organisms die during a static test or 5% during a flow-through study.

4. Beginning the Test

Static acute tests are initiated either by adding the test material to the test chambers after the freshwater invertebrates are added or by adding the invertebrates to the test chambers within 30 minutes after the test material is added to the dilution water.

5. Measuring Temperature/DO/pH

Temperature should be measured continuously (hourly) in at least one test vessel during the entire study period. If temperature is controlled by a water bath, measurements can be recorded every six hours. Temperature should not vary more than one degree Centigrade (C) during the entire study period.

The dissolved oxygen (DO) concentration must be measured at the beginning of the test and every 48 hours thereafter to the end of the test. Measurements should be taken from the control and the high, medium, and low concentrations as long as animals are present at those levels. The DO level during the first 48 hours should be between 60% and 100% of saturation and between 40% and 100% saturation after 48 hours. In the flow-through test, the DO concentrations in each chamber should be between 60% and 100% saturation at all times during the study.

The pH should be measured at the beginning and end of the test in the control and the high, medium, and low toxicant concentrations.

6. Chemical Analysis

It is preferred that solutions be chemically analyzed to determine exact concentrations of pesticides. It is particularly important that residues are measured if:

- The test solutions were aerated (aeration may cause volatilization of the pesticide);
- The test material was volatile, insoluble or precipitated out of solution;
- The test containers were not made of stainless steel or glass;
- The test chemical is known to adsorb to the test container's structural material; or
- A flow-through system is used (measurement verifies accuracy of metering system).

III. REPORTING REQUIREMENTS

The test report submitted to the Agency must fully describe the materials and methodology used to perform the study. The reviewer must be able to establish from the report that the study was performed under conditions that render the results acceptable for use in a risk assessment and/or for fulfilling the guidelines

requirement. The following information is particularly important for a complete evaluation.

A. Test Material

If the study is to be performed with the technical grade product, the test material should be clearly identified as to source, batch, and exact purity. Simply identifying the material as technical may not be acceptable because the percent active ingredient of some newer products may increase with time as the manufacturing process is improved to produce greater yield.

For studies involving the end-use product, the exact percent of the active ingredient and the type of formulation (e.g., granular, wettable powder) of the test material should be described. It should be clearly stated in the test report whether results are expressed in terms of active ingredient or as total formulated product.

B. Dilution Water/Test Vessels

Test reports submitted to the Agency should include a complete description of dilution waters used in the toxicity studies. Descriptions should include identification of the source, the chemical characteristics of the water, and information on any pretreatments.

Test containers should be described as to construction materials, size, diluent depth, and volume.

C. Test Organisms

Test reports should provide complete descriptions of source, holding, and acclimation conditions including information on feeding schedules and disease treatment procedures.

Age, size and/or life stage of organisms should reported. Species should be identified by scientific name.

D. Range Finding Tests

Test reports should provide information describing range finding study procedures and results. The information should include sample sizes, concentrations tested, and mortality data.

E. Definitive Tests

Procedures used to prepare toxicant stock solutions test material aliquots should be thoroughly described. Dosing methods should be reported.

The criteria for determining effects must be defined. The raw data or percentage of deaths/effects at each level as well as the

number of freshwater invertebrates tested per level must be reported for each 24-hour period of the study. Toxic symptoms (physical and behavioral) should be described throughout the test period.

F. Calculated LC50

The statistically calculated LC_{50} with 95% confidence limits and the method of calculation must be presented. The slope of the dose-response line should be calculated and reported.

In lieu of a calculated LC_{50} (or EC_{50}), the study may show that the LC_{50} (EC_{50}) is greater than 100 ppm.

G. Temperature/DO/pH

Dissolved oxygen and pH measurements should be reported along with the range and average temperature.

H. Chemical Analyses

If chemical analyses are conducted, the test report should provide information on the methods (or references to established methods) utilized and results of analyses. Residues found at the beginning and end of the study should be reported.

I. Testing Protocols

The test report should include references to any protocols followed during the test.

IV. REVIEWER'S EVALUATION

A. Review of Test Conditions

The reviewer should note any important information missing from the submitted report. Also noted are conditions of the study that are inconsistent with recommended methodologies as discussed in this SEP or in designated references.

B. Verification of Statistical Analyses

An integral part of the data evaluation process is the verification of statistical analyses. The reviewer should ensure that the LC_{50} has been properly derived by recalcuating data through currently available statistical programs.

An acceptable acute toxicity test should provide additional important information other than the LC_{50} (EC_{50}). Results from

An acceptable acute toxicity test should provide additional important information other than the LC_{50} (EC_{50}). Results from a valid study should provide a zero mortality level and a slope of the dose-mortality response line. These data can give further insight into the toxicological characteristics of the chemical such as whether the response is gradual over a wide concentration range or relatively rapid over a narrow range.

If the recalculated results differ substantially from the submitted results, the reviewer should note this and attempt to explain the discrepancies.

A test can be considered unacceptable if more than 10% of the control organisms die during the study period. An inadequate number of test organisms per test level can also produce unreliable results.

C. Conclusions

1. Categorization of Results

The significance of inconsistencies in the test procedures must be determined by the reviewer so that the results of the study can be categorized as to their usefulness in a risk assessment. Categories are described as:

° Core: All essential information was reported and the study was performed according to recommended protocols. Minor inconsistencies with standard metholdologies may be apparent; however, the deviations do not detract from the study's soundness or intent. Studies within this category fulfill the basic requirements of Part 158 of the regulations and are acceptable for use in a risk assessment.

° Supplemental: Studies in this category are scientifically sound; however, they were performed under conditions that deviated substantially from recommended guideline protocols. Results do not meet regulatory requirement; however, the information may be useful in a risk assessment.

Some of the conditions that may place a study in a supplemental category include:

- Unacceptable test species;
- Inappropriate test material;
- Dosage levels tested were less than 100 ppm but not high enough to produce an effect on the organisms or a precise LC_{50} (EC_{50}); or
- Deviations from recommended test solution characteristics (variations in DO, temperature, hardness, and pH can affect toxicological response).

° Invalid: These studies provide no useful information. They are not scientifically sound, or they were performed under conditions that deviated so significantly from recommended protocols that the results will not be useful in a risk assessment.

Examples of studies placed in this category commonly include those where the test system was aerated, test vessels were constructed from materials other than glass, or there were problems of solubility or volatility of the test material. Unless acceptable chemical analyses of actual toxicant concentrations were performed in studies such as these, the reviewer cannot be sure that organisms were actually exposed to nominally designated residues. Also, a study where the test material was not properly identified can be invalidated.

2. Rationale

To support a supplemental or invalid category, the reviewer must list and explain all test conditions that deviated from standard protocols.

3. Repairability

If any or all of the deviations can be re-examined and found acceptable (i.e., the study category can be upgraded), the reviewer also discusses this. Usually to upgrade a study additional information must be acquired.

D. Descriptive Classification

Valid aquatic LC_{50} (EC_{50}) toxicity values can be categorically compared to LC_{50} (EC_{50}) values determined for other chemicals and/or species by the following descriptive classification:

LC_{50} (EC_{50}) (ppm)	Category Description
< 0.1	very highly toxic
$0.1 - 1$	highly toxic
$> 1 \leq 10$	moderately toxic
$> 10 \leq 100$	slightly toxic
> 100	practically non-toxic

These descriptive categories are for inter-chemical comparisons only and do not reflect actual environmental hazard to freshwater organisms.

E. References

The reviewer should reference any information used in the validation procedure. This should include protocol documents, statistical methods, or information taken from files of other division branches.